Dysbiosis-induced IL-33 contributes to impaired antiviral immunity in the genital mucosa

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Commensal microbiota are well known to play an important role in antiviral immunity by providing immune inductive signals; however, the consequence of dysbiosis on antiviral immunity remains unclear. We demonstrate that dysbiosis caused by oral antibiotic treatment directly impairs antiviral immunity following viral infection of the vaginal mucosa. Antibiotic-treated mice succumbed to mucosal herpes simplex virus type 2 infection more rapidly than water-fed mice, and also showed delayed viral clearance at the site of infection. However, innate immune responses, including type I IFN and proinflammatory cytokine production at infection sites, as well as induction of virus-specific CD4 and CD8 T-cell responses in draining lymph nodes, were not impaired in antibiotic-treated mice. By screening the factors controlling antiviral immunity, we found that IL-33, an alarmin released in response to tissue damage, was secreted from vaginal epithelium after the depletion of commensal microbiota. This cytokine suppresses local antiviral immunity by blocking the migration of effector T cells to the vaginal tissue, thereby inhibiting the production of IFN-γ, a critical cytokine for antiviral defense, at local infection sites. These findings provide insight into the mechanisms of homeostasis maintained by commensal bacteria, and reveal a deleterious consequence of dysbiosis in antiviral immune defense.

commensal microbiota | dysbiosis | IL-33 | herpes simplex virus type 2 | genital tract

Commensal microbiota perform various immunologic functions in mammals, including controlling the development of lymphoid tissue, immune cell homeostasis, and the resistance to invasive pathogens (1, 2). In particular, the role of commensal microbiota in the regulation of antiviral immunity has been widely studied. For example, commensal microbiota have been shown to provide immune inductive signals for antiviral protection (3, 4). Commensal microbiota supply signals for inflammasome activation (3), and calibrate IFN responsiveness by providing tonic signals (4). On the other hand, commensal microbiota are known to suppress antiviral immunity by promoting viral entry and replication (5–7). However, how commensal bacteria influence the effector arm of antiviral immunity has not been studied.

Belkaid and colleagues recently reported the critical role of commensal bacteria residing in barrier surfaces, such as skin for local immunity against invasive pathogens (8). According to this study, local resident commensals, rather than gut commensal microbiota, provide signals supporting a microenvironment conducive to protective immunity. The female genital tract, composed of distinct upper and lower parts, is one such barrier surface. The lower genital tract, the vaginal mucosa in particular, is both the site of pathogen entry and the first line of defense against pathogens. As with other barrier surfaces, commensal microbiota, largely of the *Lactobacillus* species, shape the microenvironment of the vaginal tract. Lactobacilli produce hydrogen peroxide to prevent the outgrowth of harmful bacteria, and maintain the acidic pH of the vaginal mucosa through lactic acid production, which provides resistance against pathogens (9). Imbalances in the vaginal flora, called dysbiosis, result in outgrowth of pathogenic bacteria; however, the consequence of dysbiosis on genital antiviral immunity remains to be elucidated.

Genital herpes is a frequently relapsing, chronic viral disease. It is not only one of the most common sexually transmitted viral infections, but also a significant risk factor for other sexually transmitted infections, such as HIV-1. Although several studies in humans have described the association between bacterial vaginosis and genital herpes simplex virus (HSV) infection (10, 11), the mechanisms through which dysbiosis modulates antiviral immunity to HSV infection remain unclear.

To this end, we investigated the mechanisms by which commensal bacteria elicit immune protection against HSV-2 infection of the vaginal mucosa. We demonstrate that dysbiosis within the vaginal microbiota leads to a dramatic increase in IL-33, which in turn blocks the ability of effector T cells to migrate into the vaginal tissue and secrete the antiviral cytokine IFN-γ, thereby rendering the host susceptible to lethal infection by HSV-2.

Results

Defective Immune Protection Against Mucosal HSV-2 Infection in Antibiotic-Treated Mice. Although there have been several studies investigating the role of commensal bacteria in antiviral immunity,

Significance

Protective mechanisms of commensal bacteria against viral infection are limited to how immune inductive signals are provided by commensal bacteria for enhancing immunity. Whether, or how, commensal bacteria might influence the effector arm of immune responses remains unknown. Here, we demonstrate that dysbiosis within the vaginal microbiota results in severe impairment of antiviral protection against herpes simplex virus type 2 infection. IL-33 released into the vaginal tract after antibiotic treatment blocks the ability of effector T cells to migrate into the vaginal tissue and secrete the antiviral cytokine, IFN-γ. Thus, our findings suggest a previously unstudied role of commensal bacteria in the effector phase of the antiviral immune response against genital herpes.

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results vary depending on type of virus and route of exposure. For example, commensal microbiota provide immune inductive signals for antiviral immunity against mucosal influenza virus or systemic lymphocytic choriomeningitis virus (LCMV) infections, whereas commensal microbiota promote viral infection by murine leukemia virus or poliovirus (3, 4, 6, 7). To determine the importance of commensal bacteria in defense against mucosal infection by HSV-2, we examined the outcome of intravaginal infection with 10^4 pfu of WT HSV-2 after administration of a combination of five oral antibiotics for 4 wk. All mice treated with antibiotics died within 11 d of viral infection, but some of the animals in the control treatment group survived HSV-2 infection (Fig. 1*A*). Moreover, antibiotic-treated mice exhibited more severe pathology than control mice (Fig. 1*B*). We observed no difference in viral titers from the vaginal secretion or vaginal tissue at early points after infection; however, viral titers in the vagina remained significantly elevated in antibiotic-treated mice at later time points (Fig. 1*C*). It has been reported that administration of a Toll-like receptor agonist mimicking the effect of commensal bacteria can restore immunity in antibiotic-treated mice (3, 4, 12). Thus, we inoculated mice locally (intravaginal) or distally (intrarectal or oral) with LPS before mucosal HSV-2 infection. Unlike previous studies, neither local nor distal LPS treatment enhanced the survival of antibiotic-treated mice following HSV-2 infection (*SI Appendix*, Fig. S1*A*). Taking these data together, we find that depletion of commensal bacteria results in a severe defect in antiviral protection following mucosal HSV-2 infection, and administration of LPS to mimic commensal bacteria cannot recover protective immunity following mucosal HSV-2 infection in antibiotic-treated mice.

Antibiotic Treatment Induces an Imbalance in Microbial Composition of the Vaginal Mucosa. In vaginal ecosystems, a low diversity of bacterial communities (dominated by *Lactobacillus* species) seems to represent a healthy vaginal flora (13, 14). In contrast, low diversity of the gut microbiota has been considered as a causative factor for inflammatory bowel disease (15). To assess the effects of antibiotic treatment on bacterial load and composition, stool and vaginal washes were collected from control and antibiotic-treated mice. Microbiological analyses indicate that antibiotic treatment results in a significant decrease in bacterial density, predominantly diminishing Gram-positive bacteria, in both the intestinal lumen and vaginal mucosa (Fig. 2*A*). In addition, the amount of 16S rDNA present in the stool and vagina was significantly decreased in antibiotic-treated mice, consistent with a reduced bacterial load (Fig. 2*B*). By using 16S ribosomal DNA sequencing, we identified the change in composition of commensal microbiota inhabiting the vaginal mucosa after 4 wk of antibiotic treatment. Consistent with our microbiological analysis, a portion of Gram-positive bacteria (e.g., *Firmicutes*) was decreased, whereas Gram-negative *Proteobacteria* was increased after antibiotic treatment (Fig. 2*C*). Assessment of vaginal microbiota revealed that bacterial composition diversified after treatment, and these compositional changes started as early as week 1 after antibiotic treatment (*SI Appendix*, Fig. S2*A*). Furthermore, in antibiotic-treated mice these bacteria formed a cluster that was distinct from that of controls (*SI Appendix*, Fig. S2*B*). Collectively, these data show that although the amount of bacterial colonization was diminished, various species of bacteria different from that of control mice colonized in vaginal mucosa after antibiotic treatment.

Next, to investigate which bacterial classes are responsible for immune protection against mucosal HSV-2 infection, we treated mice with a single type of antibiotic. Treatment with any antibiotic alone did not induce the same level of severe mortality or morbidity as treatment with all of the antibiotics combined (*SI Appendix*, Fig. S1 *B* and *C*). These results indicate that not just one class of commensal bacteria takes responsibility for immune protection against mucosal HSV-2 infection. Instead, it is the imbalance of vaginal commensal bacteria that renders antibiotic-treated mice susceptible to mucosal HSV-2 infection, because only treatment with five oral antibiotics, not any single type of antibiotic, induced severe mortality and morbidity after HSV-2 infection.

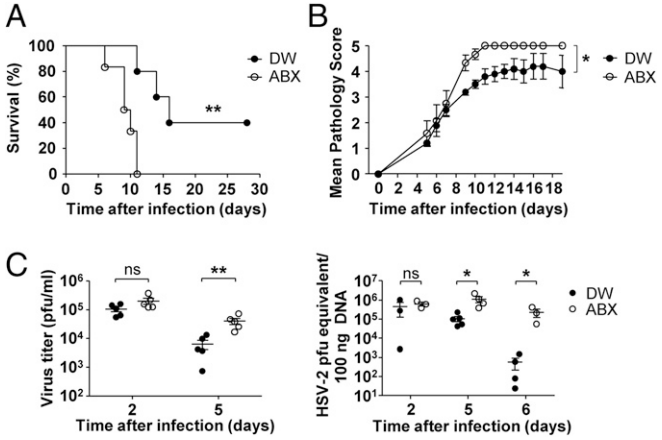

Fig. 1. Commensal microbiota control immune protection against mucosal HSV-2 infection. C57BL/6 mice were given ampicillin, vancomycin, neomycin sulfate, gentamicin, and metronidazole (ABX) in drinking water or were given DW as a control for 4 wk before intravaginal WT HSV-2 infection (10^4 pfu per mouse). Survival (*A*) and disease scores (*B*) were assessed for 1 mo postchallenge ($n = 5$ mice). Similar results were obtained from five independent experiments. (*C*, *Left*) At the indicated days postinfection, HSV-2 viral titers from vaginal washes were measured on Vero cells. (*Right*) Genome copy number of HSV-2 gB DNA isolated from vaginal tissues was determined by PCR. Data are representative of two independent experiments ($n = 3$–5 mice per group). *$P < 0.05$; **$P < 0.01$; ns, not significant. Error bars: SEM.

Innate Immunity to Mucosal HSV-2 Infection Is Not Affected by Antibiotic Treatment. To investigate how commensal microbiota support immune protection against HSV-2 infection in the vaginal mucosa, we first explored the effect of commensal bacteria on the innate immune response. Innate immunity plays a pivotal role in limiting viral replication and initiating adaptive immunity in response to primary viral infection (16, 17). Following vaginal mucosal HSV-2 infection, production of proinflammatory cytokines, such as IL-6, TNF-α, and IL-12p40, was not impaired in antibiotic-treated mice (Fig. 3*A*). However, consistent with previous studies (3, 8), antibiotic treatment reduced the production of IL-1 family cytokines, such as IL-1α, IL-1β, and IL-18 (Fig. 3*B*). Notably, pro–IL-1β production by the vaginal mucosa was reduced in antibiotic-treated mice relative to control-treated mice, both at steady state and after viral infection (*SI Appendix*, Fig. S3*A*). However, treatment with IL-1β could not restore protective immunity against mucosal HSV-2 infection in antibiotic-treated mice (*SI Appendix*, Fig. S3 *B* and *C*).

Type I IFNs are centrally involved in innate antiviral defense through up-regulation of numerous IFN stimulatory genes that antagonize viral replication and activate adaptive immunity (18). Type III IFNs, such as IFN-λ, are also known to exert type I IFN-like antiviral activities, especially on mucosal epithelia (19, 20). Previously, commensal bacteria have been shown to contribute to IFN responses against systemic LCMV and respiratory influenza virus infection (4). However, we found that type I IFNs, including IFN-α and IFN-β, and type III IFN (IFN-λ) production was not impaired, rather slightly increased, following HSV-2 infection of the vaginal mucosa in antibiotic-treated mice (Fig. 3*C*).

To test whether innate effector cells trafficked normally to the site of infection following administration of antibiotics, we examined the proportion and number of effector immune cell

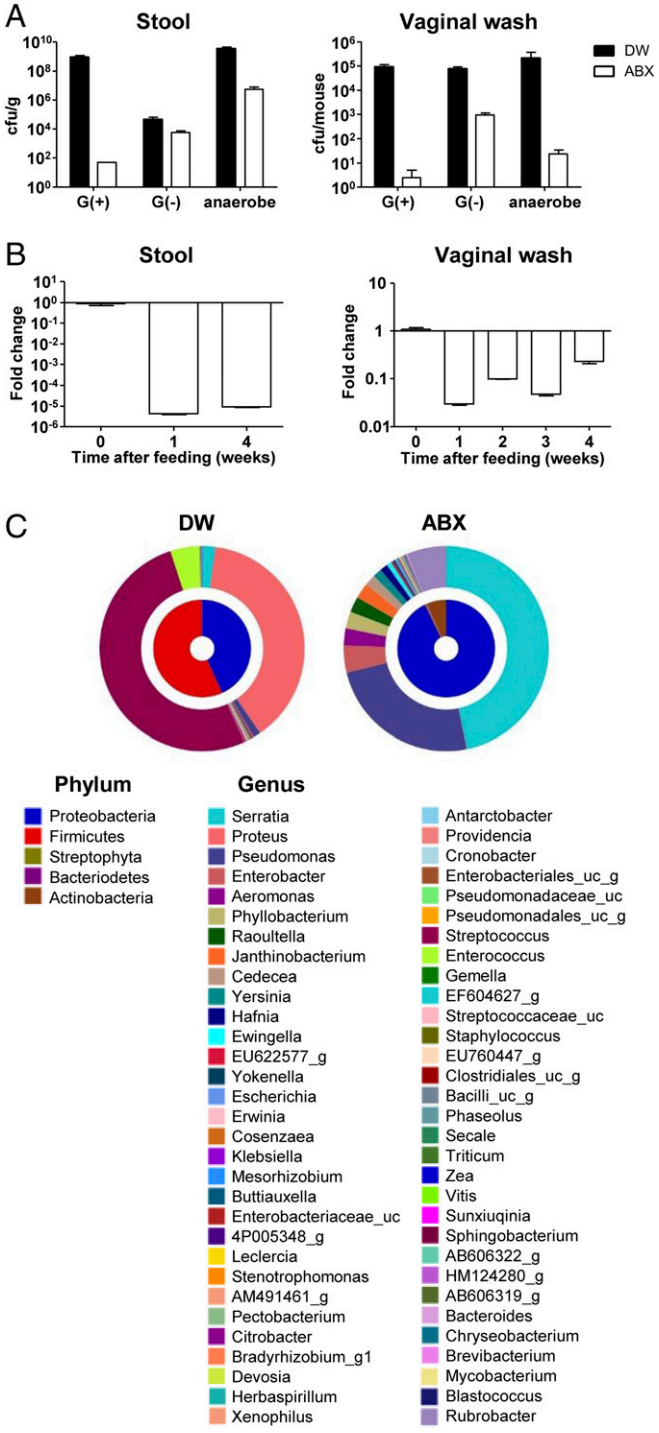

Fig. 2. Decreased bacterial colonization, but increased bacterial diversity, after antibiotic treatment. C57BL/6 mice were administered antibiotics in drinking water for 4 wk. (*A*) Bacterial loads in stools and vaginal washes pooled from five mice were measured. Data are a compilation of three experiments. Error bars: SEM. (*B*) Stool and vaginal wash pellets pooled from six mice were collected before (week 0) or during (weeks 1–4) antibiotic treatment. Relative gene copies of 16S rDNA isolated from stool and vaginal wash pellets were quantified by qPCR. Error bars represent SEM of technical duplicates. Similar results were obtained from two independent experiments. (*C*) Double pie charts demonstrating the microbial communities residing in vaginal washes collected from DW or ABX mice ($n = 10$ mice pooled per group). Inner pie: composition of phyla; Outer pie: composition of genera. Similar results were obtained from two independent experiments.

subsets, including dendritic cells, plasmacytoid dendritic cells, inflammatory monocytes, neutrophils, and eosinophils in the vaginal tissue early after infection (Fig. 3*D*). Our data demonstrate that both the proportion and number of eosinophils were markedly increased regardless of infection, whereas other innate immune cells migrated similarly to the site of infection in antibiotic-treated and control-treated mice. Collectively, these results suggest that the innate immune response to mucosal HSV-2 infection is not impaired following treatment with antibiotics. Instead, antibiotic treatment induces an increase in tissue eosinophils, even in the absence of viral infection.

Impaired Recruitment of Effector T Cells to the Site of Infection Despite Normal T-Cell Priming. Next, we evaluated the possibility that commensal bacteria contribute to adaptive immunity against mucosal HSV-2 infection. In HSV-2 infection, both CD4 T and CD8 T cells and the IFN-γ produced by these cells are essential for defense (21). However, no significant changes in the frequency of IFN-γ–producing CD4 or CD8 T cells were found in either draining lymph nodes or vaginal tissues after antibiotic treatment (*SI Appendix*, Fig. S4). Moreover, T-cell priming in draining lymph nodes after vaginal mucosal HSV-2 infection was not impaired in antibiotic-treated mice (Fig. 4*A*).

Despite the lack of a defect in T-cell priming, it is possible that immune defense might be impaired at the vaginal mucosa because of defective migration of effector T cells from the site of immune induction to the site of viral replication (22, 23). To see whether activated T cells were properly recruited to the infection site, we measured the frequency of CD4 and CD8 T cells in vaginal tissue and found that the frequency of these cells and the number of activated T cells, defined as $CD44^{+}CD62L^{-}$, in vaginal tissues were markedly reduced in antibiotic-treated mice after infection (Fig. 4*B*). Moreover, the level of IFN-γ, a crucial cytokine for local protection against mucosal HSV-2 infection, was markedly reduced in vaginal washes on day 5 after HSV-2 infection of antibiotic-treated mice. Furthermore, CXCL9, which induces recruitment of T cells to the infection site in response to IFN-γ, was decreased as well. In contrast, the level of CXCL10, which plays a similar role to CXCL9, was comparable between the two groups, likely because CXCL10 is also induced in response to other types of IFNs (24) (Fig. 4*C*).

In addition, we investigated whether a shorter duration of antibiotic treatment could affect immune responses against mucosal HSV-2 infection because the amount and the compositional changes in vaginal microbiota start as early as 1 wk after treatment. Interestingly, a 2-wk treatment with antibiotics also resulted in an impairment in immune protection, leading to poor survival and severe pathology (*SI Appendix*, Fig. S5*A*). Notably, we also observed higher viral titers in the vagina of antibiotic-treated mice than control mice at late time points, whereas viral titers at early times did not differ (*SI Appendix*, Fig. S5*B*). Similar to previous results, 2 wk of antibiotic treatment resulted in a reduced proportion and number of CD4 and CD8 T cells in vaginal tissue after viral infection (*SI Appendix*, Fig. S5*C*). Moreover, cytokines and chemokines (such as IFN-γ and CXCL9) in vaginal fluids of these mice were markedly reduced on day 5 after HSV-2 infection (*SI Appendix*, Fig. S5*D*). Together, these results indicate that defective migration of effector T cells in antibiotic-treated mice induces diminished production of IFN-γ at the site of infection in the same time frame as the amount and composition of vaginal microbiota change.

Antibiotic Treatment Induces the Secretion of IL-33, Which Recruits Type 2 Innate Lymphoid Cells and, Subsequently, Eosinophils to the Vaginal Mucosa. To investigate what influences the defects in local immune defense found under conditions that deplete commensal microbiota, we performed a proteomic analysis to identify changes in the abundance of specific vaginal wash proteins in

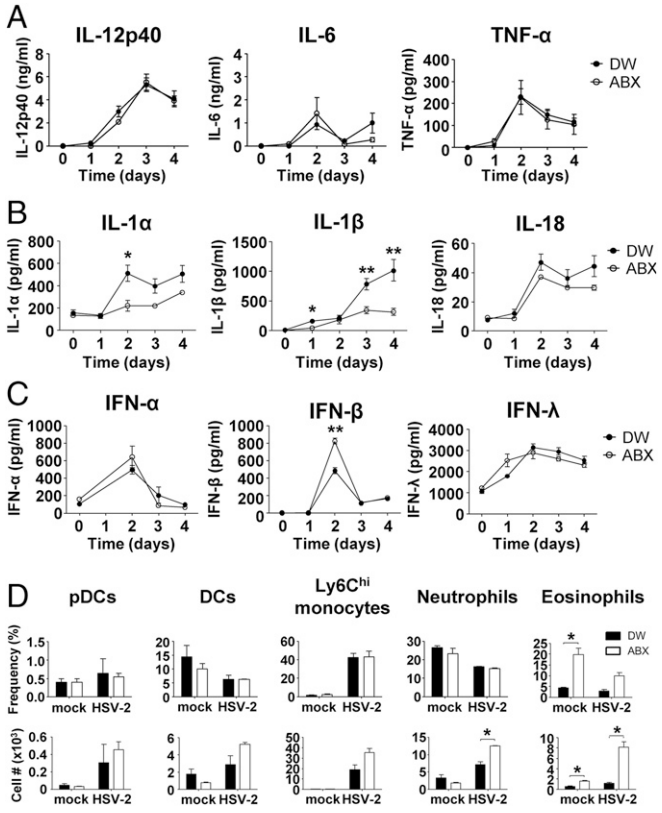

Fig. 3. Innate immune response against mucosal HSV-2 infection is not impaired in antibiotic-treated mice. C57BL/6 mice fed with antibiotics (ABX) in drinking water or DW for 4 wk were infected intravaginally with 10^4 pfu of WT HSV-2. (*A–C*) At the indicated days postinfection, levels of (*A*) proinflammatory cytokines including IL-12p40, IL-6, and TNF-α; (*B*) IL-1 family cytokines including IL-1α, IL-1β, and IL-18; and (*C*) IFNs, including IFN-α, IFN-β, and IFN-λ, were measured in vaginal washes using ELISA (DW, $n = 5$ mice; ABX, $n = 6$ mice). Similar results were obtained from two independent experiments. (*D*) At 48 h, frequency (*Upper*) and number per mouse (*Lower*) of innate immune cells in vaginal tissues were assessed using flow cytometric analysis ($n = 3$ mice). Plasmacytoid DCs (pDCs) were defined as Ly6C$^+$BST2$^+$CD11c$^+$CD11b$^-$ cells, dendritic cells (DCs) as MHCII$^+$CD11c$^+$ cells, Ly6C^{hi} monocytes as Ly6C^{high}CD11b$^+$ cells, neutrophils as Ly6G$^+$CD11b$^+$ cells, and eosinophils as Siglec-F$^+$CD11b$^+$ cells. Data are representative of three independent experiments. *$P < 0.05$; **$P < 0.01$. Error bars: SEM.

antibiotic-treated mice relative to untreated controls. Of 184 proteins identified, 26 were significantly increased (≥threefold) in vaginal wash fluid from antibiotic-treated mice relative to untreated controls (*SI Appendix*, Tables S1–S3). The level of proteins associated with tissue damage (serum albumin, S100-A8, S100-A9) and acute-phase proteins representing inflammation (ceruloplasmin, ferritin, α-1-antitrypsin) was increased in vaginal wash fluid. Consistently, the level of albumin was also found to be increased in vaginal wash fluid after antibiotic treatment when measured by ELISA (Fig. 5*A*). On the basis of these results, we hypothesized that factors driven by inflammatory damage of vaginal epithelial cells during antibiotic treatment modulate local immunity.

Based on the fact that IL-33 is one of well-known damage-associated molecules, we tried to detect IL-33 in vaginal washes of antibiotic-treated mice. Using nanoflow liquid chromatography electrospray-tandem mass spectrometry analysis, we detected a dramatic increase in IL-33 at 1 wk postantibiotic treatment (*SI Appendix*, Fig. S6). Similarly, the level of IL-33 measured by ELISA was also increased in the vaginal wash fluid over the course of antibiotic treatment (Fig. 5*B*). Meanwhile, other cytokines related to innate (IL-1α, TNF-α, IL-6) and adaptive (IFN-γ, IL-4) immunity, as well as epithelial-derived cytokines (IL-25, TSLP), did not increase during antibiotic treatment (*SI Appendix*, Fig. S7).

IL-33 is well known for its involvement in Th2-related immune responses. The receptor for IL-33 consists of ST2 (also known as IL-33R), which is highly expressed on type 2 innate lymphoid cells (ILC2), and IL-1R accessory protein (IL-1RAcP) (25). Upon activation by IL-33, ILC2 release type 2 cytokines, such as IL-5 and IL-13, and this IL-33–mediated ILC2 activation promotes

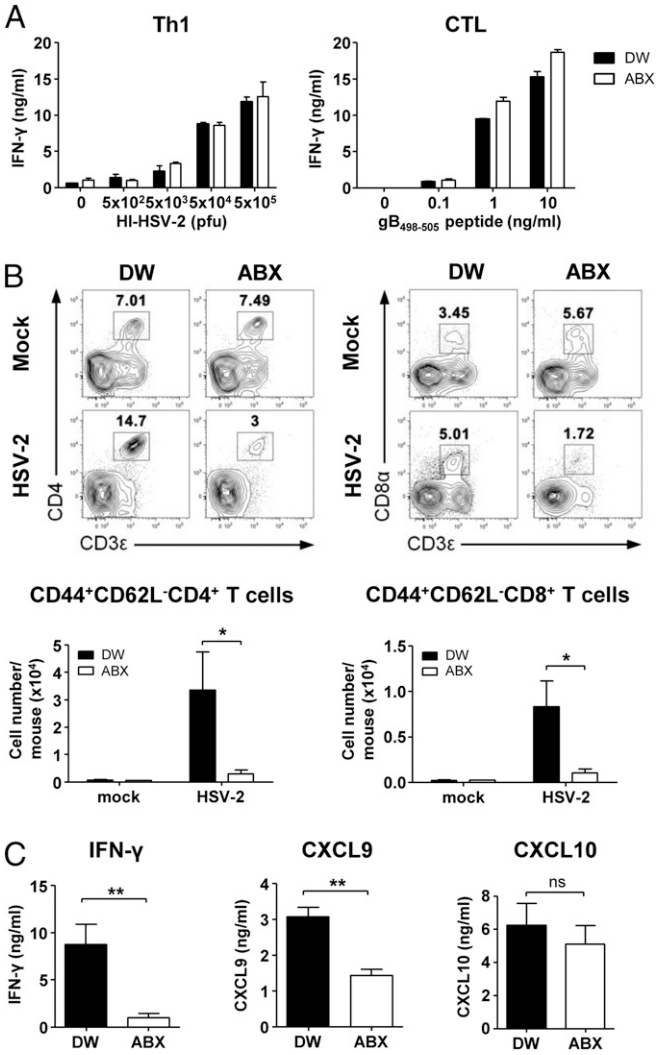

Fig. 4. IFN-γ production at the local infection site is impaired after mucosal HSV-2 infection of antibiotic-treated mice. C57BL/6 mice fed with antibiotics (ABX) in drinking water or DW for 4 wk were infected intravaginally with HSV-2. (*A*) At day 6 postinfection with 10^6 pfu of TK-HSV-2, IFN-γ produced by CD4 or CD8 T cells isolated from draining lymph nodes after restimulation with heat-inactivated HSV-2 or gB peptide for 72 h was measured using ELISA ($n = 3$ mice). Data are representative of three independent experiments. (*B*) At day 6 postinfection with 10^4 pfu of WT HSV-2, the recruitment of CD4 and CD8 T cells to vaginal tissues was assessed using flow cytometry. Plots were gated on DAPI$^-$CD45.2$^+$ cells and numbers indicate the percentage of gated cells. The numbers of activated (identified as CD44$^+$CD62L$^-$) CD4 T and CD8 T cells were assessed (mock, $n = 4$ mice; HSV-2, $n = 7$ mice). Data are a compilation of three experiments. (*C*) Levels of IFN-γ and the IFN-inducible chemokines CXCL9 and CXCL10 were measured in vaginal washes at day 5 postinfection with 10^4 pfu of WT HSV-2 using ELISA (DW, $n = 5$ mice; ABX, $n = 6$ mice). Data are representative of three independent experiments. *$P < 0.05$; **$P < 0.01$; ns, not significant. Error bars: SEM.

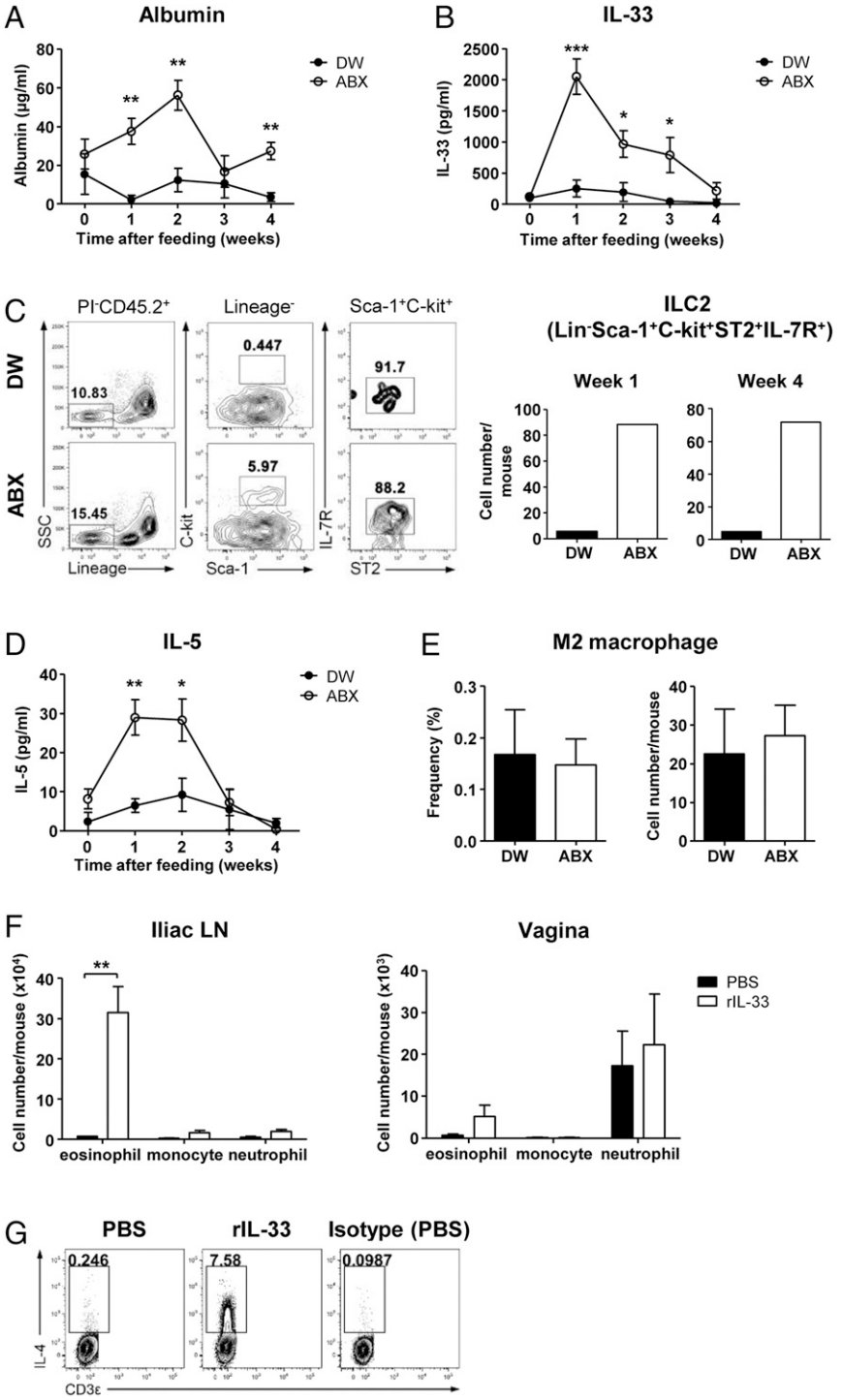

Fig. 5. IL-33 secretion, which promotes Th2-related immune responses, is induced in vaginal mucosa after antibiotic treatment. (*A–E*) C57BL/6 mice were administered antibiotics (ABX) in drinking water or DW for 4 wk. (*A*) Albumin level was measured by ELISA from vaginal washes during antibiotic treatment (DW, $n = 5$ mice; ABX, $n = 6$ mice). (*B*) Level of IL-33 was measured in vaginal washes collected during the course of antibiotic treatment by ELISA (DW, $n = 5$ mice; ABX, $n = 6$ mice). (*C*, *Left*) After 4 wk of antibiotic treatment, the frequency of lineage (CD3ε, B220, CD11b, CD11c, NK1.1, and FcεRI)$^-$Sca-1$^+$C-kit$^+$ST2$^+$IL-7R$^+$ innate lymphoid cells in vaginal tissues was analyzed by flow cytometry. Numbers indicate percentage of gated cells. (*Right*) The number of innate lymphoid cells in vaginal tissues after 1 wk and 4 wk of antibiotic treatment was assessed (DW, $n = 5$ mice pooled; ABX, $n = 6$ mice pooled). (*D*) Level of IL-5 was measured in vaginal washes collected during the course of antibiotic treatment by ELISA (DW, $n = 5$ mice; ABX, $n = 6$ mice). (*E*) Frequency and number of M2 macrophages in vaginal tissues were assessed using flow cytometric analysis (DW, $n = 4$ mice; ABX, $n = 5$ mice). M2 macrophages were defined as CD206$^+$ F4/80$^+$CD11b$^+$ cells. (*F* and *G*) C57BL/6 mice were injected intraperitoneally with recombinant IL-33 or PBS daily for 8 d. (*F*) Iliac lymph nodes and vaginal tissues were collected, and the number of immune cells analyzed by flow cytometry was graphed ($n = 4$ mice). Eosinophils were defined as Siglec-F$^+$CD11b$^+$ cells, monocytes as Ly6C^{high}CD11b$^+$ cells, and neutrophils as Ly6G$^+$CD11b$^+$ cells. (*G*) Six days after final injection, IL-4 production by CD3ε$^-$CD4$^-$CD8α$^-$ cells from iliac lymph nodes was measured by intracellular cytokine staining after stimulation with PMA and ionomycin ($n = 3$ mice). Data are representative of two to three independent experiments $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Error bars: SEM.

recruitment of innate cells involved in type 2 responses, including eosinophils (26, 27). To reveal the mechanism through which IL-33 acts and identify the cells affected by this cytokine in our study, we determined which cells expressed ST2 in vaginal tissues before HSV-2 infection. We discovered that ILC2, which express Sca-1, C-kit, ST2, and IL-7R, but not hematopoietic lineage markers, are

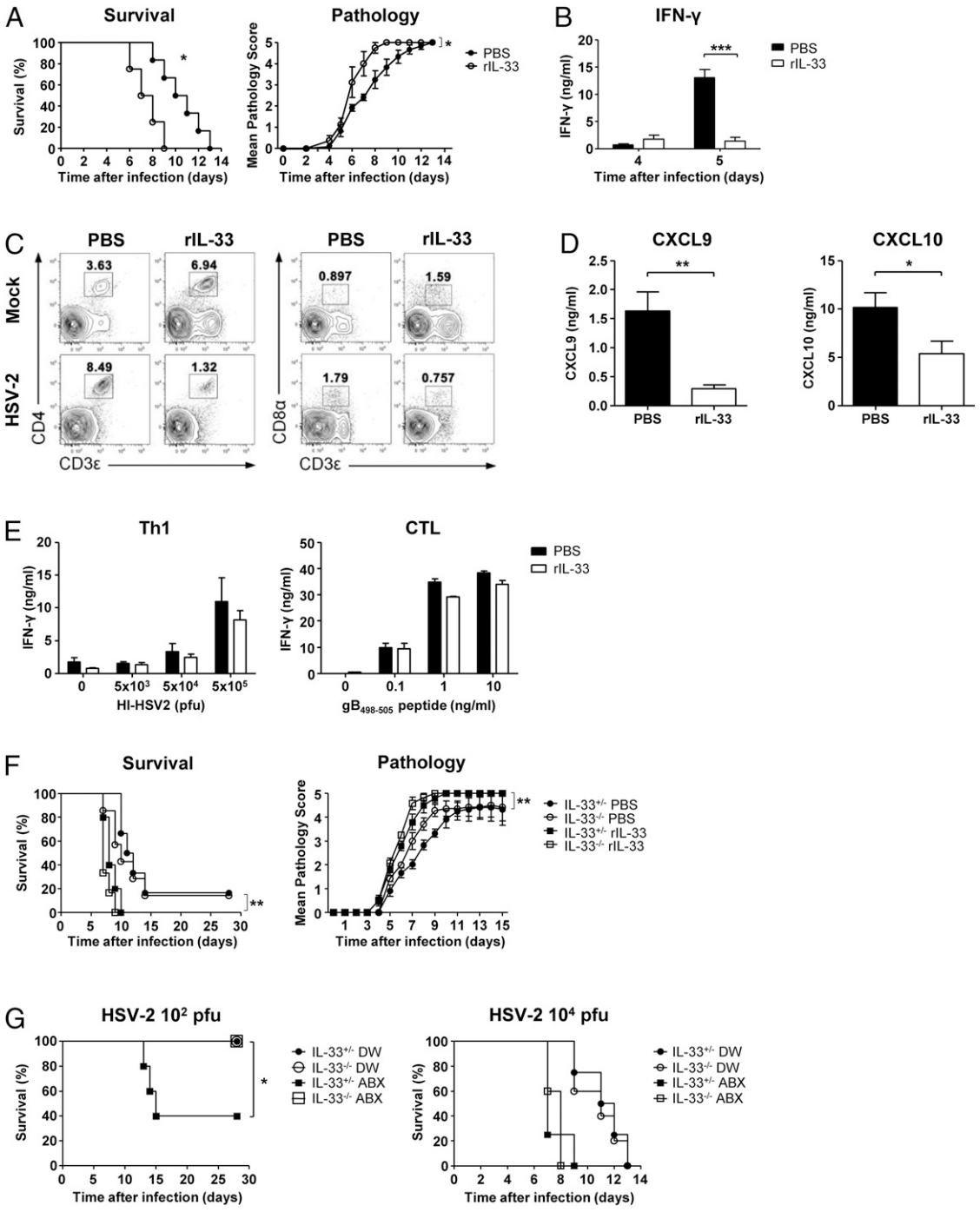

Fig. 6. IL-33 suppresses immune protection against mucosal HSV-2 infection. (*A–E*) C57BL/6 mice were injected intraperitoneally with recombinant IL-33 (2 μg per mouse) or PBS daily for 8 d. Immediately after the final injection, mice were infected intravaginally with 10^4 pfu of WT HSV-2. (*A*) Survival and disease scores were monitored, and (*B*) vaginal washes were collected on the indicated days postinfection to measure IFN-γ by ELISA (PBS, $n = 5$ mice; rIL-33, $n = 6$ mice). (*C*) At day 6 postinfection, the recruitment of CD4 and CD8 T cells to vaginal tissues was assessed by flow cytometry. Plots were gated on $DAPI^-CD45.2^+$ cells and numbers indicate the percentage of gated cells ($n = 3$ mice per group). (*D*) Vaginal washes were collected at 5 d postinfection to measure CXCL9 and CXCL10 by ELISA (PBS, $n = 5$ mice; rIL-33, $n = 6$ mice). (*E*) At day 6 postinfection, IFN-γ produced by CD4 or CD8 T cells isolated from draining lymph nodes after restimulation with heat-inactivated HSV-2 or gB peptide for 72 h was measured by ELISA ($n = 3$ mice). (*F*) $IL\text{-}33^{+/-}$ or $IL\text{-}33^{-/-}$ mice were injected intraperitoneally with recombinant IL-33 or PBS daily for 8 d. Immediately after the final injection of IL-33, mice were infected intravaginally with 10^4 pfu WT HSV-2. Survival and disease scores were monitored ($n = 5$–7 mice per group). (*G*) $IL\text{-}33^{+/-}$ or $IL\text{-}33^{-/-}$ mice fed with antibiotics (ABX) in drinking water or DW for 4 wk were infected intravaginally with 10^2 pfu (*Left*) or 10^4 pfu (*Right*) of WT HSV-2. Survival was monitored ($n = 4$–5 mice per group). Data are representative of two independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Error bars: SEM.

present in the vaginal mucosa of antibiotic-treated mice (Fig. 5*C*). In addition, the level of IL-5 was also increased in the vaginal wash fluid over the course of antibiotic treatment (Fig. 5*D*). In the context of tissue damage after antibiotic treatment, we tried to detect alternatively activated macrophages (AAMs, M2 macrophage), which regulate the tissue repair response, in the vaginal tissue. After antibiotic treatment, however, the proportion and number of AAMs did not increase in the vaginal tissue (Fig. 5*E*). Combined with the data showing that the proportion and number of eosinophils are markedly increased in the vaginal tissue after antibiotic treatment (Fig. 3*D*), we concluded that damage-associated production of IL-33 in antibiotic-treated mice facilitates the recruitment of ILC2 into the vaginal mucosa, which leads to the production of IL-5 and accumulation of eosinophils in vaginal tissues.

IL-33 Contributes to Impaired Antiviral Immunity to Mucosal HSV-2 Infection. Next, to corroborate a role for IL-33 in modulating local immune defense against HSV-2, we injected mice with recombinant IL-33 (rIL-33) every day for 8 d before viral infection. Mice injected with rIL-33 exhibited eosinophil infiltration into the iliac lymph nodes and vaginal mucosa (Fig. 5*F* and *SI Appendix*, Fig. S8). Moreover, IL-4 was produced from non-B and non-T cells in the iliac lymph nodes of these mice, even at steady state (Fig. 5*G*). After HSV-2 infection, mice injected with rIL-33 died much faster and exhibited more severe pathology than control mice (Fig. 6*A*). These mice were unable to produce IFN-γ in vaginal mucosa at 5 d postinfection (Fig. 6*B*), as shown in antibiotic-treated mice. Furthermore, in mice injected with rIL-33, the recruitment of effector T cells to the site of infection was defective (Fig. 6*C*), although T-cell priming in the draining lymph nodes was not impaired (Fig. 6*E*). In addition, CXCL9 and CXCL10 production from vaginal mucosa was reduced in these mice at 5 d postinfection (Fig. 6*D*). Notably, mice treated locally with rIL-33 also showed impaired survival and more severe pathology than control mice (*SI Appendix*, Fig. S9*A*), which suggests that the local effect of IL-33 is sufficient to suppress protective immunity against mucosal HSV-2 infection.

IL-33 is known to drive protective, antiviral cytotoxic T lymphocyte responses in cases of systemic LCMV infection (28). However, IL-33–deficient mice exhibited a survival rate and pathology score comparable to those of control mice in response to vaginal mucosal HSV-2 infection (*SI Appendix*, Fig. S9*B*). Moreover, CD4 and CD8 T-cell–derived IFN-γ production was normal in the draining lymph nodes and vagina of IL-33–deficient mice (*SI Appendix*, Fig. S9*C*). To elucidate the distinct role of IL-33 during vaginal mucosal HSV-2 infection, we injected rIL-33 into IL-33–deficient mice and then infected these mice with WT HSV-2 intravaginally. Like IL-33 heterozygous mice injected with rIL-33, IL-33–deficient mice injected with rIL-33 were susceptible to mucosal HSV-2 infection (Fig. 6*F*). Moreover, IL-33–deficient mice treated with antibiotics showed comparable survival rates with untreated mice (distilled water, DW) when infected with a low dose of HSV-2; however, these mice could not overcome the defect in protection caused by antibiotic treatment when infected with a high dose of HSV-2 (Fig. 6*G*). Taken together, these findings suggest that IL-33 could be a key factor in the suppression of antiviral immunity following mucosal HSV-2 infection through the inhibition of local IFN-γ production as a result of defective migration of effector T cells to local infection sites.

Proteases, Such as Those Induced by Dysbiosis, Induce IL-33 Secretion in the Vaginal Mucosa and Lead to Impaired Antiviral Immunity to Mucosal HSV-2 Infection. Commensal microbiota confer host protection from damage by exogenous pathogens (29). Thus, we hypothesized that IL-33, an alarmin released from dying cells, may be induced by damage caused by pathogens grown out after antibiotic treatment. By analyzing 16S ribosomal sequencing data, we found that the relative abundance of *Serratia* and *Pseudomonas* was increased in antibiotic-treated mice (Fig. 7*A* and *SI Appendix*, Fig. S2*C*). Based on the evidence that some species of *Serratia* and *Pseudomonas* are able to produce proteases (30–32), especially cysteine protease (33–35), we investigated whether IL-33 is secreted in vaginal mucosa after treatment with papain, a cysteine protease. Although papain treatment mimics an acute response and antibiotic treatment leads to a long-term effect on vaginal commensals, we discovered that IL-33 levels in vaginal mucosa increased after papain treatment and then gradually decreased (Fig. 7*B*). Moreover, pretreatment with active papain resulted in poor survival and severe pathology following mucosal HSV-2 infection (Fig. 7*C*). These findings suggest that protease-producing pathogenic bacteria, which grow abundantly as a result of oral antibiotic treatment, may induce IL-33 secretion in vaginal mucosa, and in turn lead to impaired antiviral immunity against mucosal HSV-2 infection.

Discussion

Our findings suggest a previously unknown effect of commensal microbiota on the local tissue microenvironment during the effector phase of defense against viral infection. Antibiotic-mediated depletion of commensal microbiota impaired antiviral immunity, which resulted in severe morbidity and mortality and poor control of viral replication following mucosal HSV-2 infection.

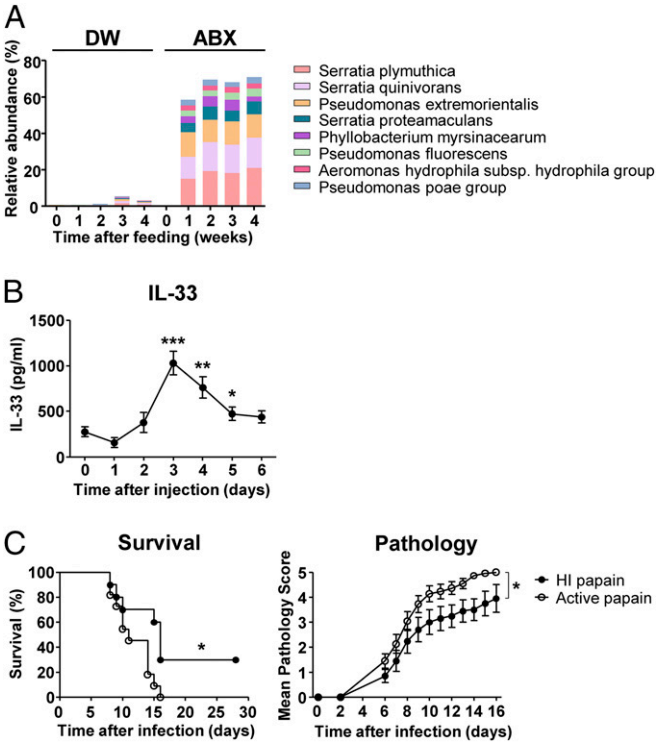

Fig. 7. Proteases, such as those induced by dysbiosis, induce IL-33 secretion into the vaginal mucosa, and lead to impaired antiviral immunity against mucosal HSV-2 infection. (*A*) Bar plots indicate relative abundance of bacterial species that predominate in ABX mice ($n = 6$ mice) over DW mice ($n = 5$ mice). (*B*) IL-33 was measured by ELISA of vaginal washes collected after intravaginal injection of the protease papain for 3 consecutive days ($n = 11$ mice). Data are representative of two independent experiments. (*C*) C57BL/6 mice were injected intravaginally with active papain or heat-inactivated (HI) papain for 3 consecutive days. At 4 d after final injection, mice were infected intravaginally with 10^3 pfu of WT HSV-2. Survival and disease scores were monitored for 4 wk postchallenge ($n = 10$–11 mice per group). Data are representative of two independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Error bars: SEM.

Furthermore, not only did antibiotic treatment reduce commensal microbiota, it also increased the antibiotic-resistant microbiota, resulting in an imbalance in the microbial composition of the vaginal tract. The susceptibility of antibiotic-treated mice was attributed to compromised effector T-cell responses rather than defects in innate immunity. Specifically, we found that IL-33 secreted from damaged epithelia recruited ILC2 and subsequently eosinophils into vaginal tissue, which contributed to suppression of antiviral immunity against mucosal HSV-2 infection. Finally, protease activity induces increased levels of IL-33 in the vaginal tract, suggesting that proteases produced by some antibiotic-resistant bacterial species may exert similar effects in vivo. Taken together, these results indicate that dysbiosis-induced IL-33 contributes to the impaired antiviral immunity against mucosal HSV-2 infection.

The role of commensal microbiota in the regulation of antiviral immunity has been studied previously (3, 4). In these studies, commensal microbiota provided immune inductive signals for inflammasome activation or calibration of IFN responsiveness. Therefore, depletion of commensal microbiota resulted in defective immune responses against mucosal and systemic virus infections. In contrast, our results suggest that dysbiosis rather than reduction of commensal microbiota are more likely to contribute to impairment of protective immunity against mucosal HSV-2 infection. Recent human study also supports that high diversity vaginal microbiome correlates with high susceptibility to genital infections (14). Inoculation of Toll-like receptor agonists to reconstitute the effects of commensal bacteria could not restore immune responses in our study, but this treatment completely restored antiviral immunity in other studies (3, 4). In addition, treatment with single antibiotics was previously found to reduce immune responses (3), whereas we found that treatment with any antibiotic alone did not induce the same level of mortality or morbidity as treatment with a combination of antibiotics. Collectively, these findings suggest that impaired protection against mucosal HSV-2 infection in antibiotic-treated mice is not a consequence of a reduction in commensal bacteria or depletion of a specific group of bacteria. Instead, the effect of antibiotic-resistant microbiota predominated in the vaginal microenvironment renders antibiotic-treated mice susceptible to mucosal HSV-2 infection.

IL-33 is one of several epithelial cell-derived cytokines (including IL-25 and TSLP), and functions as an alarmin released in response to tissue damage. Although epithelial barrier tissues and endothelial cells are generally known to express IL-33 (36, 37), some immune cells, such as macrophages and dendritic cells, can produce IL-33 under certain inflammatory conditions (38, 39). However, the level of IL-33 produced by hematopoietic cells is much less than that from epithelial cells (39, 40), and tissue-derived IL-33 is critical for induction of allergic inflammation (41). IL-33 signals through the ST2/IL–1RAcP complex, mainly expressed in ILC2, mast cells, basophils, eosinophils, Th2 cells, NKT, and NK cells (25, 42, 43). Notably, ILC2, one of the initial targets of IL-33 because of their constitutive expression of ST2, mediates type-2 immunity through secretion of type 2 cytokines, such as IL-5 and IL-13. These cytokines induce tissue eosinophilia, goblet cell hyperplasia with increased mucus production, and recruitment and activation of AAMs and Th2 cells, which together induce and reinforce type 2 immunity (44). In our study, antibiotic treatment markedly increased IL-33 secretion from vaginal epithelia, which induced the recruitment of ILC2s into vaginal tissue. In addition, the level of IL-5 was also increased in parallel with IL-33, and eosinophils infiltrated into vaginal tissue in antibiotic-treated mice. Although the exact mechanism through which IL-33 impedes the migration of IFN-γ–producing T cells remains to be determined, we assume that IL-33 acts locally on ILC2 to induce the secretion of type 2 cytokines and subsequent recruitment of eosinophils into vaginal tissue, thereby inhibiting the secretion of IFN-γ from recruited T cells. This process would result in a reduction of IFN-induced chemokines CXCL9 and CXCL10 and, as a result, a reduction in further recruitment of effector T cells.

In our study, exogenous IL-33 dramatically suppressed antiviral immunity against mucosal HSV-2 infection by blocking the ability of IFN-γ–producing T cells to migrate into the vaginal tissue. However, antibiotic-treated mice were still susceptible to HSV-2 infection even in the absence of IL-33, when infected with a lethal dose of HSV-2. In contrast, antibiotic-treated IL-33–deficient mice survived comparable to water-fed control mice when they were infected with a sublethal dose of HSV-2. Unlike exogenous IL-33 treatment, antibiotic treatment could induce various changes affecting host immune responses besides IL-33 secretion from vaginal epithelia. Although changes in antibiotic-treated mice, including diminished production of IL-1 family cytokines, might contribute to impaired protective immune responses (3, 8), IL-33 was found to be sufficient to suppress antiviral immunity against genital HSV-2 infection. However, in the absence of IL-33 signals, antibiotic-mediated immunologic changes could render these mice susceptible to HSV-2 infection, although this suppression could be overcome by the host immune system at lower viral titers. Thus, we concluded that IL-33 induced by antibiotic treatment might be a dominant modulator suppressing host antiviral immunity against mucosal HSV-2 infection.

Although previous studies have shown that stimulatory signals from commensal microbiota are crucial for defense against viral infection (3, 4), our present study demonstrates that inhibitory signals induced by the depletion of commensal microbiota also affect antiviral immunity. Taken together, our findings provide a unique insight into the role of commensal bacteria in maintaining the integrity of surface barrier epithelial cells by preventing pathogenic bacteria colonization, thereby supporting a microenvironment conducive to antiviral defense. These results also prompt clinically relevant questions regarding the use of oral antibiotics and the potential for increased susceptibility to various sexually transmitted viruses.

Materials and Methods

Mice. Female 6- to 8-wk-old C57BL/6 specific pathogen-free mice were purchased from DBL Co. Ltd, Korea. IL-33$^{-/-}$ mice (45) were kindly provided by the RIKEN Center for Developmental Biology. All mice were housed in a specific pathogen-free facility of the Korea Advanced Institute of Science and Technology (KAIST). All procedures involving animals were in accordance with the guidelines and protocol (KA2013-55) for rodent experimentation provided by the Institutional Animal Care and Use Committee of KAIST.

Antibiotic Treatment. Mice were treated with ampicillin (500 mg/L; AG Scientific), vancomycin (250 mg/L; AG Scientific), neomycin sulfate (500 mg/L; AG Scientific), gentamicin (500 mg/L; AG scientific), and metronidazole (500 mg/L; Sigma) in drinking water for 2 or 4 wk, as previously described (4). Antibiotic treatment was started 2 or 4 wk before infection and continued for the duration of the experiment. For some experiments, individual antibiotics (at the indicated concentrations) were administered in drinking water.

Virus and Intravaginal Infection. The WT and TK- HSV-2 strains provided by A. Iwasaki (Yale University, New Haven, CT) were used for all experiments. HSV-2 was propagated and titrated using a plaque assay on Vero cells. For intravaginal virus infection, mice were injected subcutaneously with medroxyprogesterone acetate (Tokyo Chemical Industry) at 2 mg per mouse in 100 μL at 5–7 d before infection, swabbed with calcium alginate, and inoculated intravaginally with 10^2 to 10^4 pfu WT or 10^6 pfu TK- HSV-2 in 10 μL using a blunt-ended micropipette tip, as previously described (46). Upon WT HSV-2 challenge, disease severity was scored as follows (47): 0, no sign; 1, slight genital erythema and edema; 2, moderate genital inflammation; 3, purulent genital lesions; 4, hind-limb paralysis; 5, premoribund. Because of humane concerns, the animals were killed before reaching the moribund state.

Vaginal Viral Titration and Measurement of Cytokines and Albumin. Vaginal fluids were collected from 0 to 6 d after infection by pipetting PBS (50 μL) into and out of the vagina 20 times. Viral titers were measured by titrating vaginal fluids on Vero cells for 72 h in duplicate, as described previously (48). Levels of IL-1β, IL-12p40, IL-6, IL-5 (BD Biosciences), IL-4, IFN-γ, TNF-α, IFN-α, IFN-β, IL-33, IL-25, TSLP (eBioscience), IL-1α (BioLegend), IFN-λ, CXCL9, CXCL10 (R&D Systems), IL-18 (MBL Co., Ltd) and albumin (Immunology Consultants Laboratory) in vaginal fluids were measured using an ELISA kit according to manufacturer's instructions.

Detection of HSV-2 Glycoprotein B DNA by Quantitative PCR. After infection, vaginal tissue was collected and total genomic DNA was extracted according to the manufacturer's instructions (Qiagen). HSV-2 was measured using primers against glycoprotein B (gB) (forward: 5′-CACCGCTACTCCCAGTTTATG-3′; reverse: 5′-CGGTGGTCTCCATGTTGTT-3′) by quantitative PCR (qPCR; Bio-Rad). DNA purified from WT HSV-2 was used as the standard to calculate plaque-forming unit equivalents.

Treatment with Recombinant IL-33. rIL-33 was purchased from BioLegend. For systemic treatment, mice were injected intraperitoneally with 2 μg of rIL-33 daily for 8 d before intravaginal virus infection, as previously described, with modifications (28). For local treatment, mice were injected intravaginally with 4 μg of rIL-33 daily for 5 d before intravaginal virus infection after anesthesia with intraperitoneal ketamine (100 mg/kg) and xylazine (5.83 mg/kg) injection (49).

CD4 and CD8 T-Cell Responses. HSV-specific T-cell responses were analyzed as previously described (46). At day 6 postinfection, CD4 and CD8 T cells were isolated from the draining lymph nodes of mice infected intravaginally with 10^6 pfu of TK- HSV-2 using anti-CD4– or anti-CD8–conjugated microbeads (Miltenyi Biotech) according to the manufacturer's instructions. Next, 1×10^5 CD4 and CD8 T cells were restimulated with various amounts of heat-inactivated HSV-2 or HSV gB peptide (SSIEFARL) for 72 h at 37 °C. IFN-γ production in supernatants was measured by ELISA.

Flow Cytometry. Single-cell suspensions were prepared from the iliac lymph nodes and vagina according to previously described methods, with slight modifications (48). Single cells were pretreated with anti-CD16/32 antibody (2.4G2) to block Fc receptors, and then stained with the following antibodies: CD8α (53-6.7), B220 (RA3-6B2), Ly6C (AL-21), Ly6G (1A8), CD45.2 (104), CD11c (HL3), CD11b (M1/70), CD44 (IM7), Siglec-F (E50-2440), and NK 1.1 (PK136) (BD Biosciences); CD3ε (145-2C11), CD4 (GK1.5), CD11c (N418), CD317 (BST-2, eBio927), CD62L (MEL-14), FcεRI (MAR-1), Sca-1 (D7), c-kit (2B8), and IL-7R (CD127, A7R34) (eBioscience); MHCII (M5/114.15.2), and ST2 (DIH9) (BioLegend). Leukocytes were gated on the basis of forward and side-scatter properties, and live cells were gated on the basis of DAPI (Invitrogen) or propidium iodide (PI) exclusion. For M2 macrophage staining, single cells isolated from vagina were pretreated with anti-CD16/32 antibody (2.4G2), and surface-stained with F4/80 (BM8) (eBioscience), CD11b (M1/70) (BD Biosciences), CD115 (AFS98), CD11c (N418), and CD45.2 (104) (BioLegend). Then, cells were fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences), and intracellular stained with CD206 (C068C2) (BioLegend). Leukocytes from the iliac lymph nodes and vagina of infected mice were cultured in the presence of 50 ng/mL phorbol myristate acetate (PMA) (Sigma-Aldrich) and 1 μg/mL ionomycin (Sigma-Aldrich) for 5 h, and 2 μM GolgiStop (BD Biosciences) was added for the final 2 h. Cells were surface-stained with CD3ε (145-2C11), and CD4 (GK1.5) (eBioscience), CD8α (53-6.7), and CD44 (IM7) (BD Biosciences), and then fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. For intracellular cytokine staining, APC-labeled anti-mouse IFN-γ antibody (XMG1.2; BD Biosciences) or PE-labeled anti-mouse IL-4 antibody (11B11; BD Biosciences) was used. Live cells were gated using Ghost Dye (TONBO biosciences) for intracellular staining. All samples were acquired on an LSR Fortessa cell analyzer (BD Biosciences). All data were analyzed using FlowJo (Treestar).

Intravaginal Papain Injection. Mice were injected subcutaneously with medroxyprogesterone acetate (Tokyo Chemical Industry) at 2 mg per mouse in 100 μL at 7 d before injection, swabbed with calcium alginate, and inoculated intravaginally with 200 μg active papain or heat-inactivated (95 °C, 45 min) papain (Calbiochem) for 3 consecutive days after anesthesia with intraperitoneal ketamine (100 mg/kg) and xylazine (5.83 mg/kg) injection.

Statistical Analysis. Data are expressed as the mean ± SEM. Differences between groups on individual time points were analyzed using the unpaired, two tailed Student's *t* test. Disease scores were analyzed by two-way ANOVA test. Differences in survival were evaluated using the Log-Rank test. Statistical analysis was performed using GraphPad Prism software (GraphPad). Differences were considered statistically significant when $P < 0.05$, and are indicated as follows: $*P < 0.05$; $**P < 0.01$; and $***P < 0.001$.

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